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Assay for Estrogen and Progesterone Receptors of Breast Cancer Cell Lines in Monolayer Culture*

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Abstract—*A whole-cell assay for measuring estrogen (ER) and progesterone (PgR) receptors in monolayer culture of human breast cancer cell lines is described. It is based on the measurement of incorporated tritiated ligands during* 50 min *of incubation (i.e.* [^{3}H]*estradiol for ER,* [^{3}H]*ORG-2058 for PgR). The assay fulfills all criteria of specificity as shown by competitive studies and measurements of the dissociation constants of the binding reactions. Moreover, a subcellular fractionation of MCF-7 labeled cells revealed that the majority of incorporated steroids was associated with the nuclear fraction. This finding is consistent with the concept of nuclear location of steroid-receptor complexes. Cultures in the presence of* 10^{-8} M *estradiol indicated that the methodology is adequate for detecting the well-known estrogenic induction of PgR synthesis. The assay proved suitable for the quantitative assessment of the receptor content of various neoplastic (MCF-7; ZR-75-1, Cama-1, Evsa-T) and non-neoplastic (HBL-100) cell lines. The methodology has the other advantages of being simple and rapid, of requiring small amounts of cells and of allowing histological examination of the latter before, during and after biochemical analysis.*

INTRODUCTION

MONOLAYER cultures of human breast cancer cell lines are widely used to investigate their hormone sensitivity. They are also of considerable interest in the study of the molecular mechanism of action of steroid hormones and antagonists. Steroid receptor assays appropriate to such cultures are required for these purposes.

During the last 10 yr biochemical assays have been designed to measure estrogen and progesterone receptors in mammary tumors samples [1]. These assays, based on the measurement of the binding capacity of cellular extracts for tritiated estrogen and progestin, were extended to mammary tumor cell lines [2]. To eliminate possible artifacts due to cell disruption, methods were introduced for evaluating the uptake of tritiated steroids by cells adhering to glass scintillation vials [3] or in suspension [4]. These experimental conditions are, however, quite different from the usual culture conditions. We therefore adapted these methods to monolayer cultures. The assay that we developed was found suitable for the quantitative assessment of the receptors content of various neoplastic and non-neoplastic cell lines. It offers the additional advantage of maintaining the culture in an appropriate form for further histological and histochemical examination. It is our purpose to describe our methodology here.

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MATERIALS AND METHODS

Steroids

[^{3}H]Estradiol ([^{3}H]E_2) (90 Ci/mmol) and [^{3}H]ORG-2058 (47 ci/nmol) were purchased from Amersham, U.K. With the exception of ORG-2058, which was obtained from Amersham, all unlabeled steroids were from the Sigma Co., St Louis, MO.

Culture medium and flasks

Earle's minimal essential medium (MEM), fetal calf serum and L-glutamine were purchased from Gibco (Glasgow, U.K.); penicillin and streptomycin from Difco (Detroit, Mich.); and gentamycin from Schering (Kenilworth, NY). T-75 flasks and multiwell dishes were purchased from Falcon (Becton Dickinson).

Cell lines

The MCF-7 cell line was kindly provided by Dr M. Rich (Michigan Cancer Foundation, Detroit, MI), Evsa-T and ZR-75-1 by Dr M. E. Lippman (National Cancer Institute, Bethesda, MD) and Cama-1 by Dr J. Fogh (Sloan Kettering Institute for Cancer Research, NY). The HBL-100 line was obtained from the American Type Culture Collection (Rockville, MD). All these cell lines are maintained in our laboratory as monolayer cultures. With the exception of HBL-100, which was introduced into the laboratory recently, they were all subcultured for more than 5 yr.

Cultures were produced at 37°C in closed T-75 flasks in MEM supplemented with L-glutamine (0.6 mg/ml), gentamycin (40 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% inactivated fetal calf serum (inactivation: 1 hr at 56°C).

Uptake of [^{3}H]steroids

Cells from a confluent monolayer were removed by trypsinization (trypsin 0.05%, EDTA 0.025%) and suspended usually at 2×10^5 cells/ml in the growth medium added with 10% steroid-depleted fetal calf serum (0.5% charcoal, 0.005% dextran in 1.5 ml medium/ml of serum; overnight incubation at 4°C). The cellular suspension was then cultured in multiwell dishes (six wells per dish, 1.5 ml per well) in a humidified 95% air/5% CO_2 atmosphere. After 4 days the growth medium was replaced by serum-free medium containing [^{3}H]E_2 or [^{3}H]ORG-2058 at concentrations ranging from 0.1 to 9×10^{-9} and 0.3 to 10×10^{-9} M respectively unless otherwise specified. Additional dishes were filled with the same concentrations of [^{3}H]E_2 or [^{3}H]ORG-2058 and a 200-fold excess of the corresponding unlabeled steroid. After 10-90 min of incubation (most often 50 min; see Results) the medium was removed with a suction pump and the monolayer washed three times with ice-cold 0.9% saline. Steroids were extracted from the monolayer by a final incubation of 20 min in ethanol at room temperature. Aliquots of 200 μl of ethanol were then transferred to mini scintillation vials containing 3.6 ml of Aqualuma Plus (Lumac) for radioactivity counting. All measurements were performed in triplicate.

In each experiment an additional multiwell dish was run in parallel for DNA measurement. Cells were removed by trypsinization and DNA precipitated with 0.5 N perchloric acid. DNA was then extracted (20 min at 70°C) and measured according to the Burton method [5].

Specific [^{3}H]steroid uptake was calculated from the difference of incorporated radioactivity after incubating in the absence or presence of an excess of unlabeled steroid. It was expressed in fmol (10^{-15}mol)/μg DNA.

Dissociation of [^{3}H]steroid-receptor complexes

Cells in monolayer culture were incubated for 50 min at 37°C with 0.8×10^{-9} M [^{3}H]E_2 or 1.2×10^{-9} M [^{3}H]ORG 2058 in the absence or presence of a 200-fold excess of the corresponding unlabelled steroids. After removal of the growth medium labeled cells were washed three times with ice-cold saline and thereafter maintained at 37°C in the absence or presence of unlabeled E_2 or ORG-2058 at 0.8×10^{-9} and 1.2×10^{-9} M respectively. Incorporated radioactivity was measured at various times as described above to evaluate the dissociation of the [^{3}H]steroid-receptor complexes.

RESULTS

Cellular uptake of [^{3}H]estradiol and [^{3}H]ORG-2058

In MCF-7 cells preliminary experiments revealed that the specific uptake of tritiated E_2 or ORG-2058 increased during the first 30 min of incubation. Thereafter it progressively stabilized ([^{3}H]E_2) or slowly decreased ([^{3}H]ORG-2058) (Fig. 1). An incubation time of 50 min was selected in all further experiments.

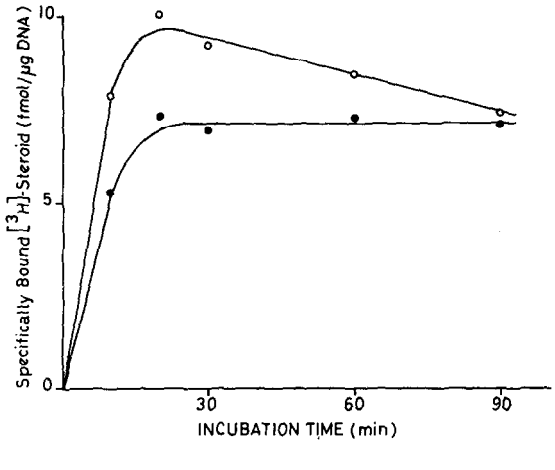

Fig. 1. Kinetics of cellular uptake of [^{3}H]E_2 (●) and [^{3}H]ORG-2058 (○) in MCF-7 cells. MCF-7 cells were incubated for various times with 8×10^{-10} M [^{3}H]E_2 or 9×10^{-10} M [^{3}H]ORG-2058. Incorporated [^{3}H]steroids were subsequently extracted and measured.

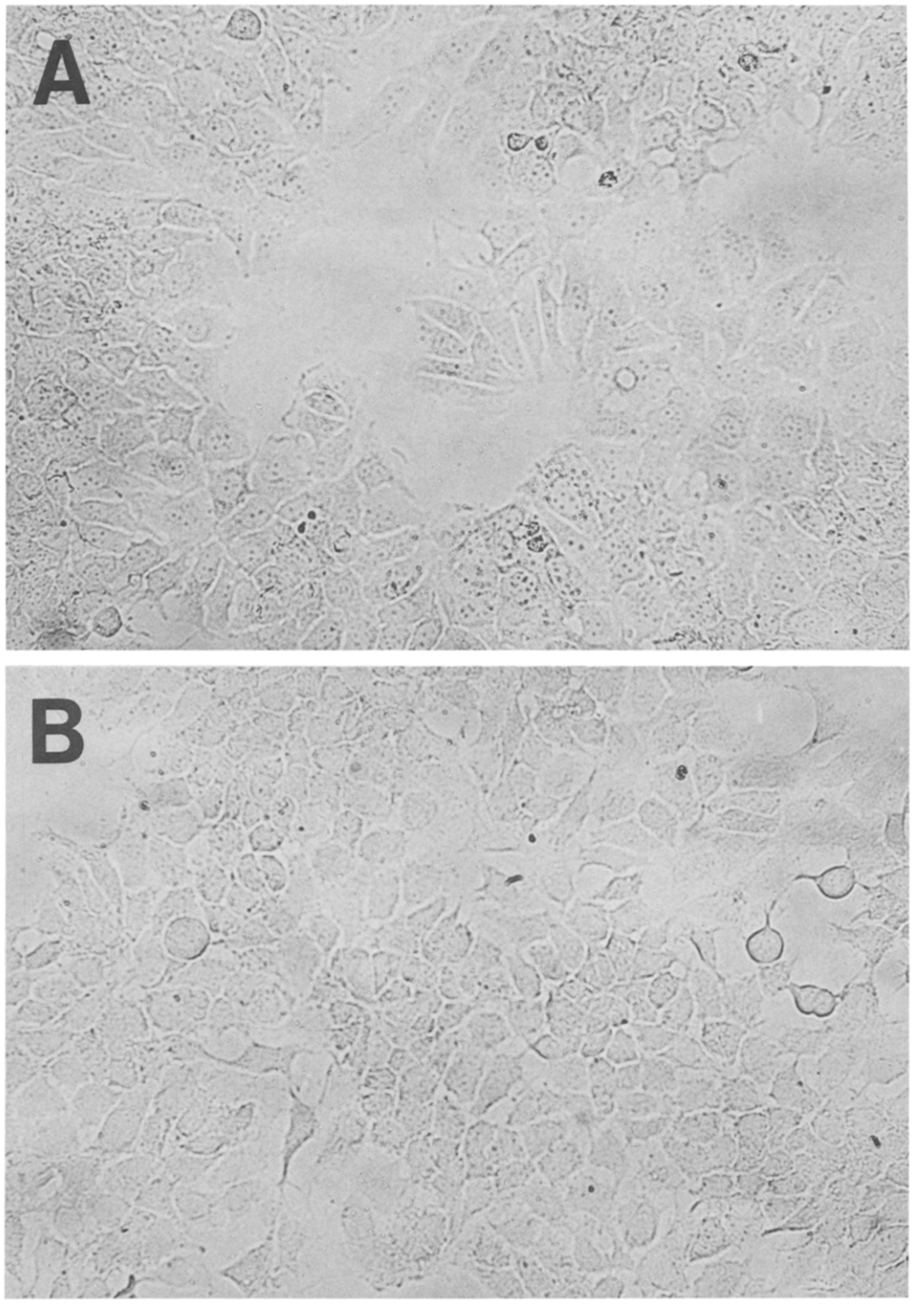

Fig. 2. Cellular aspect of MCF-7 cells before (A) and after (B) receptor assay.

Subcellular fractionation of the labeled cells according to the procedure of Pietras and Szego [6] showed that 80% of the specifically incorporated radioactivity was associated with the nuclear fraction. The remaining radioactivity was located in the microsomes and membranes; no detectable activity was found in the cytosol. On the other hand, microscopical examination of the cultures before and after ethanolic extraction of labeled steroids revealed no detectable signs of cellular alteration (Fig. 2). These results indicate that our measurements were made under adequate growth conditions.

Saturation analysis

MCF-7 cells were incubated with increasing concentrations of [^{3}H]E_2 or [^{3}H]ORG-2058 to measure the parameters of the binding reactions. Analysis of the data according to Scatchard [7] gave patterns indicative of two class of binding sites, a high-affinity class of limited capacity (slope 1) and a low-affinity class of high capacity (slope 2) (13 experiments; representative pattern in Fig. 3). These patterns, usually observed in receptor studies, are assumed to be indicative of types I (slope 1) and II (slope 2) binding sites; by definition type I corresponds to the receptors.

The dissociation constants (K_d) of the binding reactions of tritiated E_2 and ORG-2058 to the high-affinity sites were found to be $1.9 \pm 0.7 \times 10^{-10}$ and $2.9 \pm 0.2 \times 10^{-10}$ M respectively ($\bar{x} \pm$ S.D.). These values correspond to K_ds ascribed to receptors by conventional biochemical methods. Binding capacity of these sites (n) amounted to 4.0 ± 0.9 for [^{3}H]E_2 and 14.0 ± 2.3 fmol/μg DNA for [^{3}H]ORG-2058.

Specificity of the high-affinity binding sites

The specificity of the binding reactions was further studied by incubating MCF-7 cells with a constant concentration of [^{3}H]E_2 or [^{3}H]ORG-2058 in the absence or presence of increasing amounts of unlabeled estrogens, progestins, androgens and corticoids. The relative concentrations of such unlabeled potential competitors achieving a 50% inhibition of uptake gave their relative binding affinities (RBA) [8].

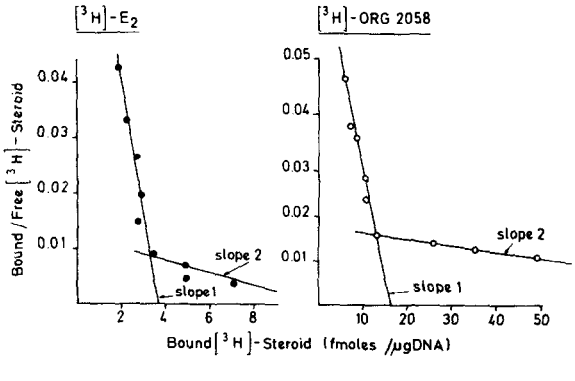

Fig. 3. Scatchard plot of [^{3}H]E_2 *and* [^{3}H]ORG-2058 *binding to MCF-7 cells.*

Estradiol, diethylstilbestrol and estrone produced a strong inhibition of [^{3}H]E_2 uptake. As expected from biochemical studies [8, 9], estradiol and diethylstilbestrol displayed a higher RBA value than estrone (Table 1). Values of all other competitors were extremely weak (<0.1%), clearly indicating that the binding of [^{3}H]E_2 was essentially limited to ER. The same conclusion was drawn for the binding of [^{3}H]ORG-2058 to PgR. Thus high RBA values were only found for the two strong progestins ORG-2058 and R-2050 (100%); all other competitors displayed weak values (<1%).

Table 1. Competitive inhibition of the binding of [^{3}H]*estradiol and* [^{3}H]*ORG-2058*

	RBA	
	[^{3}H]E_2	[^{3}H]ORG-2058
Androsterone	0.01	0.01
Cortisol	0.01	0.15
Diethylstilbestrol	100.0	0.07
Estradiol	100.0	0.80
Estrone	2.5	
ORG-2058	0.07	100.0
R-5020		100.0
Testosterone	0.03	0.6

Cells were incubated for 50 min at 37°C with 6.2×10^{-10} M [^{3}H]E_2 or 2.3×10^{-9} M [^{3}H]ORG-2058 in the absence or presence of increasing amounts of unlabeled steroids at concentrations ranging from 10^{-9} to 10^{-5} M. The incorporated radioactivity was measured to evaluable the relative concentrations of unlabeled E_2 or ORG-2058 (controls) and other steroids required to achieve a 50% inhibition of [^{3}H]E_2 or [^{3}H]ORG-2058 binding [8, 14].

$$RBA_{E_2} = ([I_{50}]\ E_2 / [I_{50}]\ \text{steroid}) \times 100;$$

$$RBA_{ORG\text{-}2058} = ([I_{50}]\ ORG\text{-}2058 / [I_{50}]\ \text{steroid}) \times 100.$$

Linearity of the assay

The uptake of [^{3}H]E_2 or [^{3}H]ORG-2058 was investigated at various stages of MCF-7 growth (from 2.5×10^4 to 1.5×10^6 cells/dish). A linear relationship was found between the amounts of DNA and of specifically incorporated [^{3}H]steroids as measured by Scatchard plot analysis (Fig. 4). This indicates the validity of the assay over a wide range of cellular concentrations (from very low to confluency).

Reversibility of binding

In MCF-7 cells removal of [^{3}H]E_2 or [^{3}H]ORG-2058 from the growth medium resulted in a progressive reduction of specifically incorporated radioactivity (Fig. 5), suggesting that bound

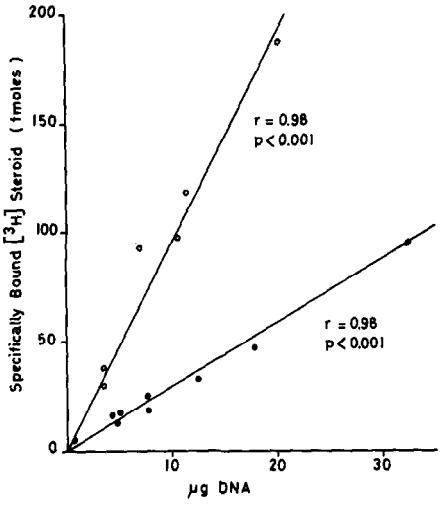

Fig. 4. Linear relation between incorporated [^{3}H]E_2 (●) *or* [^{3}H]*ORG-2058* (○) *and DNA content.*

[^{3}H]steroids may dissociate from their receptor. This phenomenon occurred at a higher rate in the presence of unlabeled E_2 or ORG-2058, revealing the possibility of an exchange process between labeled and unlabeled steroids.

Induction of progesterone receptors

E_2 is known to induce the synthesis of PgR both *in vivo* and *in vitro* [10, 11]. We investigated the validity of our methodology for detecting such an induction phenomenon. MCF-7 cells were cultured in the absence or presence of 10^{-8} M E_2 for 72 hr and the specific uptake of [^{3}H]ORG-2058 measured at various times. Figure 6 shows that a progressive increase of [^{3}H]ORG-2058 binding occurred only in the presence of E_2; after 72 hr of culture the incorported radioactivity was about three times the control value, clearly indicating the utility of the assay in the evaluation of the estrogenic stimulation of PgR.

Binding characteristics of various mammary cell lines

The extension of the present assay to other breast cancer cell lines was analyzed. Table 2 shows that, like MCF-7, the two lines recognized as being receptor-positive by biochemical methods (ZR-75-1, Cama-1) also concentrated [^{3}H]E_2 and [^{3}H]ORG-2058. The K_ds of the binding reactions were of the same order of magnitude. As expected, the receptor-negative line (Evsa-T) failed to show specific binding by the present method.

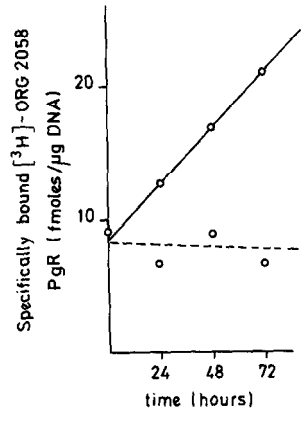

Fig. 6. Increase of [^{3}H]*ORG-2058 binding to MCF-7 cells under estradiol stimulation. The dotted line refers to the control cultures, the full lines to the cultures with* 10^{-8} M E_2.

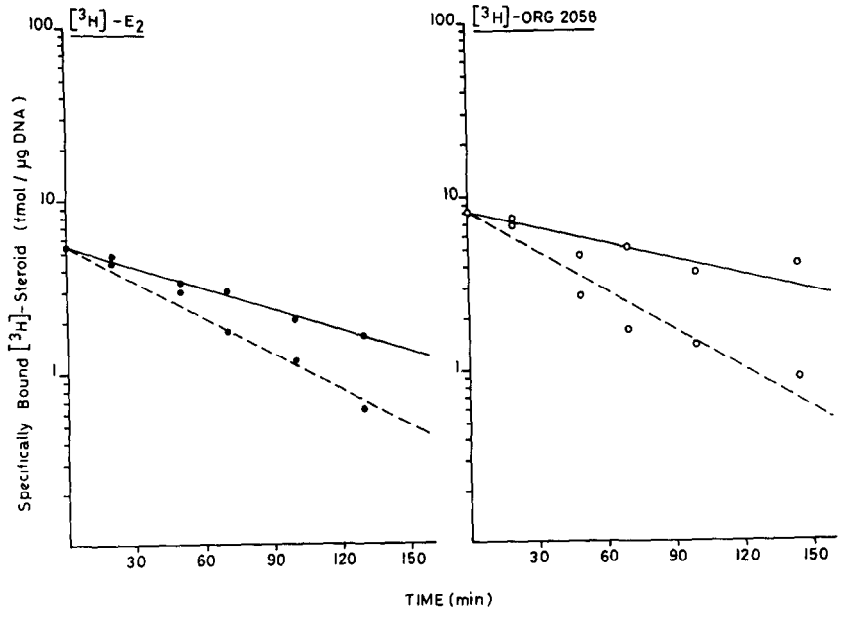

Fig. 5. Dissociation of [^{3}H]E_2 *and* [^{3}H]*ORG-2058 bound to MCF-7 cells. The full lines refer to the control medium, the dotted lines to the medium with the corresponding unlabeled steroid. Unlabeled steroids were at the same concentration as the tritiated steroids in the preceding labeling phase of the experiment* ([^{3}H]E_2, 0.8×10^{-9} M; [^{3}H]*ORG-2058*, 1.2×10^{-9} M).

Receptor-positive lines differed from each other by the amount of [^{3}H]steroid taken up, indicating different receptor concentrations (Table 2). In each line the progestin binding capacity was higher than the estrogen binding capacity. Finally, the non-cancerous line HBL 100 displayed a weak binding capacity in 2/4 analyses, suggesting that its receptor content was at the limit of detectability.

DISCUSSION

Several biochemical assays have been proposed for measuring ER and PgR in mammary tumors [1]. Almost all of them were restricted to cytoplasmic receptors unsaturated by endogenous hormones (free receptors). These assays are not suitable for breast cancer cell line analysis since some of them contain large amounts of (or only) free nuclear receptors (i.e. MCF-7, T47-D) [12, 13]. Measurement of both cytoplasmic and nuclear receptors being time-consuming, biochemical assays appear to be of limited value for investigating the receptivity of such cell lines. In addition, receptors are labile proteins which may easily be altered during cell fractionation. All of these restrictive factors favor the development of 'whole-cell assays' such as the one described here. Our data, as those reported by others [3, 4, 14-20], clearly show that the measurement of the uptake of labeled steroids by living cells gives a valuable estimation of the *total* unsaturated receptor contents.

Our methodology fulfills all criteria of specificity as shown by the competitive studies and the measurement of the dissociation constant (K_d) of the binding reactions. It is simple, rapid and requires small amounts of cells which in addition can be histologically examined before, during and after the receptor assay. This possibility of histological and histochemical assessment of the material after the analysis (i.e. cellular appearance, mitotic index. . .) presents a real interest. Finally, the assay appears especially suitable for investigating the effect of hormones and drugs on the cellular receptor concentration since it is carried out under normal growth conditions (monolayer culture). The demonstration of the estrogenic induction of PgR supports this view.

Receptors concentrations in breast cancer cell lines vary among studies [2-4, 14-20]. Table 3 shows that our own values are consistent with those reported by other investigators with whole-cell assays. Differences in growth conditions may explain the variability. Thus several authors [3, 4, 15], including us [21], here clearly shown that seric factors and hormones modulate the receptor contents of the cells. Our methodology might be a valuable tool to analyze this question.

A subcellular fractionation of labeled cells revealed that the majority of specifically incorporated steroids was associated with the nuclear fraction. This observation is consistent with the classical concept of steroid-induced translocation of the cytoplasmic receptors into the cell nucleus [22]. It also fits with the new theory that receptors are only located in the nucleus and that cytoplasmic receptors are produced during cell disruption [23, 24]. The observation reported here and elsewhere [14] that bound steroids can be displaced by free steroids under physiological conditions does not allow any opting between these two concepts. Nevertheless, it suggests a dynamic process of exchange between nuclear bound and unbound (extracellular) hormones. Turnover of the receptors [25] might be involved in this phenomenon.

The possibility of measuring the binding affinity of analogs and derivatives of labeled estrogens and progestins (competitive studies) makes our methodology appropriate for establishing structure-activity relationships. In contrast to assays carried out on cytosol [8, 9], the

Table 2. Steroid binding characteristics of various human mammary cell lines

	ER		PgR	
	n	K_d	n	K_d
MCF-7	4.0 ± 0.9†	1.8 ± 0.7(13)‡	14.0 ± 2.3	2.9 ± 0.2 (13)
Cama-1	3.1 ± 0.8	2.4 ± 1.5(4)	4.2 ± 2.3	10.0 ± 8.8 (3)
ZR-75-1	1.8 ± 0.8	0.7 ± 0.6(4)	3.7 ± 0.2	8.2 ± 7.0 (2)
Evsa-T	0	- (2)	0	- (2)
HBL-100	0.1 ± 0.0	0.7 ± 0.9 (2/4)	1.0 ± 0.8	0.3 ± 0.4 (2/4)

n = fmol/μg DNA; $K_d = 10^{-10}$ M.
†Mean value ± S.D.
‡The numbers in parentheses represent the number of experiments.

Table 3 reported estrogen and progesterone receptor concentrations

	ER		PgR	
Ref.	fmol/μg DNA	10^3 sites/cell	fmol/μg DNA	10^3 sites/cell
[14]		32 ± 5.0*		96 ± 30
[14]	3.5† 10.5‡	21 ± 4.7 † 62 ± 9.7 ‡		
[15]		62.0§ 135.0‖		
[16]		110.0		
[17]		150.0		
[18]		39.1		90.2
[3]	14.4 ± 1.0			
[19]	2.1 ± 0.3		3.4 ± 0.3	
[20]	5.8			
Our values	4.0 ± 0.9	45.1¶	14.0 ± 2.3	168

*Mean value ± S.D.
†‡Cells growing in presence or absence of bovine insuline (12.5 μg/ml).
§‖Cells plated at high (§; 10^6 cells/dish) and low (‖; 10^5 cells/dish) cell density.
¶Estimated value from DNA measurement in our culture (1 μg DNA in 5×10^4 cells).

present one provides values influenced by physiological factors such as cell entry and microsomal metabolism. Both approaches, therefore, appear complementary in drug design [26, 27].

All these considerations indicate that the whole-cell assay described here fits a large spectrum of investigations. Its introduction into routine practice should therefore be extremely helpful.

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